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**13. Abstract** ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Evidence exists that women exercising have lower estrogen levels than sedentary women. These lower estrogen levels may be the mechanism behind their reduced breast cancer risk. Previous studies included athletes with high exercise levels, and estrogen measurements were based on a few serum samples from different times during a menstrual cycle. This study included identical female twins who were discordant for moderate exercise. Estradiol was measured on a daily basis from saliva samples collected during a complete menstrual cycle. Screening interviews were initially conducted to determine eligibility and attempts were made to contact 304 pairs (reaching 272). Of these, 56 were initially eligible; however 18 declined to participate, 2 later became ineligible due to menopausal related reasons, and three only completed questionnaires. Samples proved to be un-useable for 5 pairs and another 4 pairs had an anovular cycle. Thus, results of estradiol and progesterone assays from 24 pairs with ovular cycles are provided. An index of activity level based on regular exercise as well as daily activities was developed from the daily logs. Preliminary results, based on these pairs indicated that average E2 during luteal phase was slightly higher (+7%) in the inactive member of the twin pair, while follicular phase E2 was unchanged. Average progesterone levels were slightly higher in the active member. Final results will incorporate dietary data in the analysis and control for confounding factors using multivariate analysis. Several publications are being planned.

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## 4) INTRODUCTION

The purposes of the study are the following:

(1) To determine the effect of moderate exercise on E2 levels during the follicular and luteal phases of (ovular) menstrual cycles by means of daily salivary samples in healthy premenopausal identical twins who differ in their amount of physical exercise activity per week.

(2) To determine the effect of moderate exercise on frequency of anovulation and on menstrual cycle length (specifically luteal phase length) in identical twins who differ in their amount of physical exercise activity per week.

**Overview:** Exercise has been shown to be associated with a reduced risk of breast cancer [4,5,8,10,11]. There is evidence that women exercising, for an hour or more per day, have lower serum estrogen (estradiol) levels than sedentary women (due to more anovular cycles and lower estrogen levels in ovular cycles). These lower estrogen levels appear most likely to be the mechanism behind their reduced breast cancer risk, however much is still unknown. Previous studies have, for the most part, focused on the effects of high exercise levels among athletes, as opposed to more moderate levels of exercise, on estrogen levels, and they may have been subject to 'selection bias', i.e. women who exercise may do so because of predisposing hormonal factors. In addition, the estradiol measurements have usually been based on only a few serum samples taken at different times during a menstrual cycle. This study is addressing these issues by using 60 sets of monozygous twins who are discordant with regard to moderate exercise habits (i.e. sedentary vs. exercising an average of 20 minutes/day), but are identical for heritable aspects of body build and constitution. Estradiol is being measured on a daily basis by use of salivary samples collected during a complete menstrual cycle. The subjects are being selected from pairs of healthy premenopausal identical twins under the age of 45 who participated in the California Twin Cohort Study. They are being screened to determine eligibility (i.e. neither twin having an endocrine or metabolic disorder and the pair discordant for current amount of physical exercise activity), before being asked to participate. The use of the salivary samples is an innovative method for the measurement of estradiol and offers distinct advantages over the more traditional serum hormone measurements for which daily samples are not practical. Repeated sampling, as compared to single or infrequent sampling of individuals makes it possible to more accurately characterize ovarian function and allows for a more complete assessment of estradiol levels over different phases of the menstrual cycle, without the discomfort of venipuncture or the inconvenience of office visits. Salivary steroids have been shown to be extremely stable when samples are properly treated and this method of collection is ideally suited for use in the proposed study where subjects are located throughout California[43-46]. The hormone assays are being done by Dr. Peter Ellison (Co-Investigator), an expert in the analysis of and validation of salivary samples. We are also obtaining information on daily physical exercise activity during the month of sample collection and dietary intake using established and well tested questionnaires. Analysis of covariance methods will be used to assess the relationship of estrogen levels during different parts of the menstrual cycle to exercise, controlling for diet, body

mass, and other potentially confounding factors. Based on the sample size of 60 pairs of twins, we have the power to detect differences in estradiol levels of 15% between the sedentary and moderately exercising twins. The study has important public health implications in developing strategies for the prevention of breast cancer.

**5) BODY**

Technical Objectives and Work Accomplished:

Technical objectives 1-4: Selection of twins and collection of saliva samples: Ongoing throughout Yrs. 1, 2, and 3 during the no cost extension.

1. During the course of the study identical female twins will be selected who previously participated in the California Twin Cohort and indicated that they are premenopausal.
2. These pairs will be called on the telephone and re-interviewed regarding factors related to their eligibility.
3. Once a pair is determined to be eligible and they agree to participate they will be mailed informed consent forms, saliva sample collection kits, and exercise and dietary questionnaires.
4. We will check with them periodically to determine when the first day of their period occurs and assure that they are following the directions for collection of the saliva samples.
5. They will mail their completed sample kits to Dr. Ellison's laboratory and the completed questionnaires to USC.

Work accomplished on these objectives:

We originally selected 182 identical exercise discordant female pairs from the California Twin Cohort who were born before 1957 and were part of the first group of twins sent questionnaires in 1991-1992. During 1998 questionnaires were sent to additional California Cohort twins born before 1965 and 79 pairs have been selected from this group where both members of the pair participated. During 1999-2000 questionnaires were sent to California Cohort twin born before 1973 and 43 pairs were selected for screening. Of the 304 pairs, screening interviews have been conducted with 259 (Table 1), with 32 pairs unable to be located and 13 pairs refusing the screening interview. Tracing efforts were made to no avail to locate the 32 lost pairs. From the 304 pairs, 56 (18.4%) were initially identified as eligible for the study; however 18 declined to participate (12 of these after receiving the kits) and 2 others later became ineligible due to menopausal related reasons (i.e. started taking HRT's). In addition, for five participating pairs, there were too many missing days of sample collection or other problems that caused their samples to be unuseable. Three pairs agreed to participate, but only returned the questionnaire forms and did not collect the samples. Thus there were 28 pairs for whom both members completed the study requirements; however, for 4 of these one or both members had an anovular cycle as determined by mid-luteal pooled progesterone levels (days 5-9 after day 0). Values <200 pmol/L indicated an anovular cycle.

Of those determined to be ineligible at screening, the most common reasons were use of OC's or hormones (77) followed by parity discordance (i.e. one twin parous, the other nulliparous) (31), one both twins currently or recently pregnant or breast feeding (23), and one or both twins menopausal (22).

Due to higher rates of ineligibility than anticipated we have identified fewer pairs for participation than expected. We contacted more pairs that originally planned in an effort to increase the sample size as much as possible. Despite these efforts, our final sample size was smaller that we originally had estimated. Other problems related to sample collection and anovular cycles also reduced the number of useable pairs. Some small financial compensation may have improved participation, since the requirements were demanding.

Table 1: Results of Screening Interviews and Participation

| Result of Screening of Both Members of Pair | Number of Pairs | % of Total Pairs |
|---|---|---|
| Eligible initially | 56 | 18.4 |
| Both successfully participated. Ovular cycles | (24) | (7.9) |
| Participated, one or both anovular determined by mid-luteal progesterone levels | (4) | (1.3) |
| Participated, but not useable (due to missed days, not enough volume of saliva, extremely short cycle or next period never came) | (5) | (1.6) |
| Sent questionnaires back, no sample collection | (3) | (1.0) |
| And declined all participation | | |
| Before sending kits | (6) | (2.0) |
| After sending kits | (12) | (4.6) |
| And later became ineligible (had hysterectomy, started hormones) | (2) | (0.7) |
| Screened and not eligible because: | 203 | 66.8 |
| 1+ had menopause | (22) | (7.2) |
| 1+ had very irregular periods | (3) | (1.0) |
| Parity discordant | (31) | (10.2) |
| 1 + had disqualifying disease | (8) | (2.6) |
| 1+ taking OC's or hormones | (77) | (25.3) |
| 1+ taking cortisone/prednisone | (9) | (3.0) |
| 1+ breast fed a child or pregnant within past year | (23) | (7.6) |
| Multiple of above reasons | (11) | (3.6) |
| Both had same exercise level | (18) | (5.9) |
| One twin was deceased | ( 1) | ( 0.3) |
| Lost, could not screen | 32 | 10.5 |
| Refused screening interview | 13 | 4.3 |
| Total | 304 | 100.0 |

Technical Objective 5: Completion of Hormonal Assays: Year 1, month 3 through Year 3, Month 9.

1. Dr.Ellison's Laboratory will receive the kits and will be blinded as to which twin is performing more exercise.
2. The laboratory assistant will complete the hormonal assays according to standard protocols.
3. Results will be sent to USC.

Dr. Ellison's Laboratory has processed samples from 33 pairs.. Assays have been completed for daily estradiol levels and the mean midluteal progesterone levels as well as for daily progesterone during the luteal phase (the latter assays were added to the original protocol). One member each in 4 pairs was determined to have had a non-ovulatory cycle from these results. Five additional pairs completed the collection and sent the samples to Harvard; however their samples were not useable due multiple reasons including: too many skipped days during the month, saliva samples that contained only ¼-1/3 the volume requested; cycle was abnormally short; and in one case the next cycle never came. The pair with the small volume tried a second time, but was still not able to provide a usable sample. This pair refused to use the chewing gum provided to increase saliva volume.

Technical Objectives 6-7: Data Management: Year 1, Month 6-Year 3 Month 10

1. Physical Activity questionnaires will be coded and entered at USC.
2. Dietary questionnaires will be sent to Dr. Willett for analysis, with results being sent to USC.
3. Hormonal assay data will be merged with the questionnaire data.

An Access data entry program was created for the questionnaire data and all of the received questionnaires have been entered. Data from all of the Willett dietary questionnaires completed by individual twins have been received from Harvard. We have integrated the hormonal and physical activity questionnaire data.

Technical Objectives 8-9: Data analysis and publishing of papers: Year 1, Month 12-Year 3, Month 12. And Year 4 (no-cost extension).

1. Preliminary and final analyses will be performed to address the stated hypotheses.
2. Papers will be published on the results.

A poster was presented at the Era of Hope Meeting in Atlanta with preliminary results based on the first 15 pairs with laboratory and questionnaire data available. The results have been expanded to include data from 24 pairs (with ovular cycles) and are summarized below.

**Results**: Hormone assays and questionnaires have been completed for 24 pairs with ovular cycles. The midcycle day was determined by the day of the largest drop in E2. The average daily and total follicular phase and luteal phase E2 levels were calculated. Mid luteal phase progesterone levels were used to determine if the cycle was ovular. Of the four pairs in which

one twin had an anovular cycle, in two cases it was the more active twin, in one case it was the less active twin, and in one case the activity status was undetermined. In addition there were 5 pairs with un-useable data due to problematic cycles or sample collection. In one pair, the more active twin had an extremely short cycle and in a second pair the next cycle never came for the more active twin. In two other pairs, the lesser active twin had a short cycle length or missing data and in the fifth case, both twins had too many missing days.

Both daily logs of physical activity and a general questionnaire completed prior to the sample collection were used to determine which twin was more active. The daily log was considered to be the most important since it captured activity levels during the actual time of sample collection. An index that identified the most active twin within the pair (based on regular exercise as well as physical activity during normal daily activities) was constructed as shown in Table 2.

Table 2: Index Values for determining more active twin in a pair based on size of differences between twin members for each type of activity. (Maximum points for difference=15).

| | Size of Difference between Twins in Pair |
|---|---|
| 1) Hours devoted to sports or recreation | >0.4 hrs +2 points<br>≤ 0.4 hrs +1 point |
| 2) Flights of stairs climbed | >3.7 +2 points<br>≤ 3.7 +1 point |
| 3) City blocks walked | >2.2 +2 points<br>≤ 2.2 +1 point |
| 4) % spent in vig/mod activity | >0.15% +4 points<br>.05-.15% +3 points<br>≤ .05% +2 points |
| 5) Regular vig. Activity | >0.4 +3 points<br>≤ 0.4 +2 points |
| 6) Usual pace of walking | >0.4 +2 points<br>≤ 0.4 +1 points |

The more active twin averaged significantly more hours of sports and recreational activity per week than the less active twin (4.6 vs. 1.5 hours) and had a significantly higher proportion of weekday time in vigorous or moderate activity (22.1% vs. 14.4%). The active twin was also more likely to participate in a regular exercise program at least at least once a week (69.6% vs. 45.4%).

Table 3: Physical Activity Differences Between More and Less Active Twins in a Pair

| Type of physical activity | More active twin | Less Active Twin | Paired difference |
|---|---|---|---|
| Hours of physical activity/week | 4.6 hours | 1.5 hours | -3.1*** |
| Hours of physical activity/life** | 1411.5 hours | 1250.6 hours | -160.9 |
| Flights of stairs/day | 7.3 | 5.5 | -2.0 |
| Blocks walked/day | 7.0 | 6.5 | -0.4 |
| Proportion of ave. weekday in vigorous or moderate activity*** | 22.1% | 14.4% | -8.5*** |
| Proportion of ave. weekend day in vigorous or moderate activity | 24.8% | 18.7% | -5.1 |
| Proportion getting regular exercise at least once/week | 69.6% | 45.4% | -22.7 |

***p=<.05 for difference between more active and less active twin (paired t test).

Based on 24 ovular pairs, average daily luteal phase estrogens were about 7% lower among the more active twins compared to the less active twins (22.9 vs. 24.6 pmol/L) and total luteal phase E2 was about 8.5% lower among the more active twins (280.0 vs 306.0 pmol/L) (Table 4A). In contrast, there was little difference in average daily or total follicular phase E2 by activity level (141.8 vs 136.3 pmol/L). Average progesterone levels were somewhat higher in the more active twin (178.2 vs 160.8). None of the hormone related variable differences were statistically significant. These values were calculated for a subset of these pairs that had larger intra-pair activity differences (3 points + difference based on the index) (Table 4B). Similar differences were found in this subset with the exception that the average progesterone difference was significant based on the paired t test, with the more active twin having the higher levels.

Table 4A: Hormone Values by Activity Status of Twins in 24 pairs with ovular cycles

| Hormone Assays | More Active Twin | Less Active Twin | Paired Differences |
|---|---|---|---|
| Average Luteal Phase E2/Day | 22.9 (pmol/L) | 24.6 (pmol/L) | 1.7 (p=.13) |
| Average Luteal Length[a] | 12.3 days | 12.4 days | Ns |
| Total Luteal E2 | 280.0 (pmol/L) | 306.0 (pmol/L) | 26.0 (p=.18) |
| Average Follicular Phase E2/Day | 22.4 (pmol/L) | 23.0 (pmol/L) | Ns |
| Average Follicular Length[b] | 5.8 days | 6.0 days | Ns |
| Total Follicular E2 | 141.8 (pmol/L) | 136.3 (pmol/L) | Ns |
| Ave Progesterone (luteal phase) | 178.2 (pmol/L) | 160.8 (pmol/L) | -17.4 (p=.09) |

[a] Luteal phase defined as 1 day after midcycle day until onset of next period.
[b] Follicular phase defined as 4 days after onset of period until 4 days before midcycle day.
***p=<.05 for difference between more active and less active twin (paired t test).

Table 4B: Hormone Values by Activity Status of Twins in 16 pairs with ovular cycles with larger activity differences between twins (difference in activity index 3+).

| Hormone Assays | More Active Twin | Less Active Twin | Paired Differences |
|---|---|---|---|
| Average Luteal Phase E2/Day | 22.4 (pmol/L) | 24.1 (pmol/L) | 1.8 (p=.19) |
| Average Luteal Length[a] | 12.4 days | 12.5 days | Ns |
| Total Luteal E2 | 277.4 (pmol/L) | 303.6 (pmol/L) | 26.2 (p=.29) |
| Average Follicular Phase E2/Day | 23.4 (pmol/L) | 23.4 (pmol/L) | Ns |
| Average Follicular Length[b] | 5.8 days | 5.8 days | Ns |
| Total Follicular E2 | 134.0 (pmol/L) | 139.5 (pmol/L) | Ns |
| Ave Progesterone (luteal phase)*** | 183.5 (pmol/L) | 153.9 (pmol/L) | -29.5 (p=.03) |

[a] Luteal phase defined as 1 day after midcycle day until onset of next period.
[b] Follicular phase defined as 4 days after onset of period until 4 days before midcycle day.

Among other variables compared the body mass index of the more active twin was significantly lower than that of the less active twin (23.0 vs. 23.9), but the difference was not significant (Table 5). There were no differences between the twins by age at menarche, age at first full term pregnancy or parity.

Table 5: Comparison of other variables between more and less active twin.

| Other variables | More active twin | Less active twin |
|---|---|---|
| Body Mass Index ($kg/m^2$) | 23.0 | 23.9 |
| Age at menarche | 12.7 years | 12.6 years |
| Age at first pregnancy | 22.6 years | 22.0 years |
| Number of pregnancies | 2.9 | 2.9 |

**Average Daily E2 and Progesterone Levels by Activity Level of Twin**

Figures 1 shows the average daily estradiol levels aligned by mid cycle day for the more and less active twin for the 24 pairs with ovular cycles. Slightly higher values during the luteal phase are seen for the more active twin.

**Figure 1: Mean E2 levels by menstrual cycle day for more and less active twins in 24 pairs with ovular cycles.**

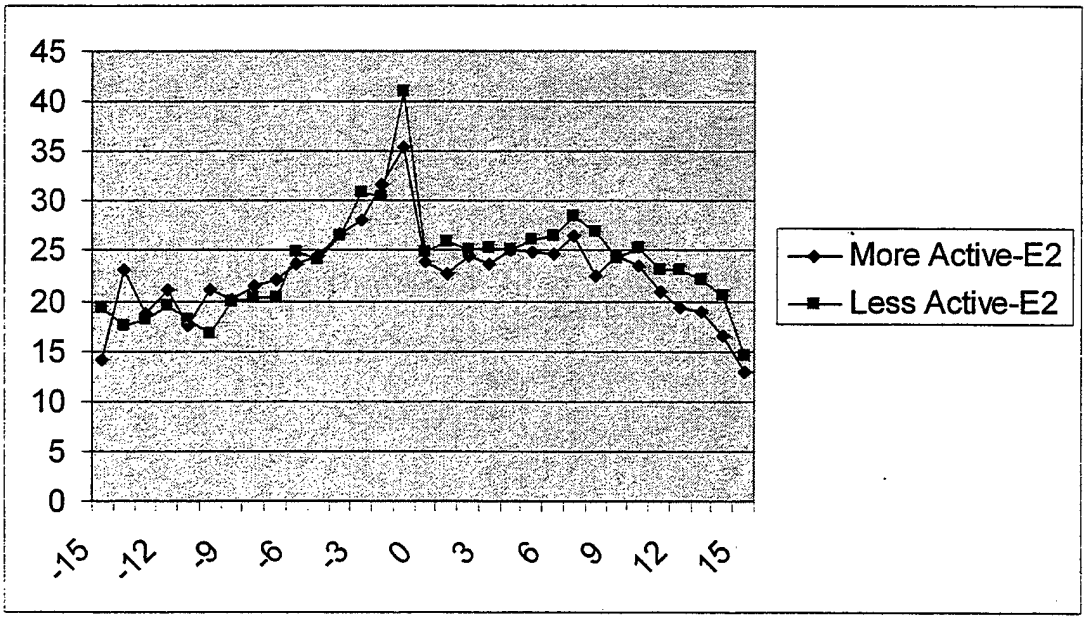
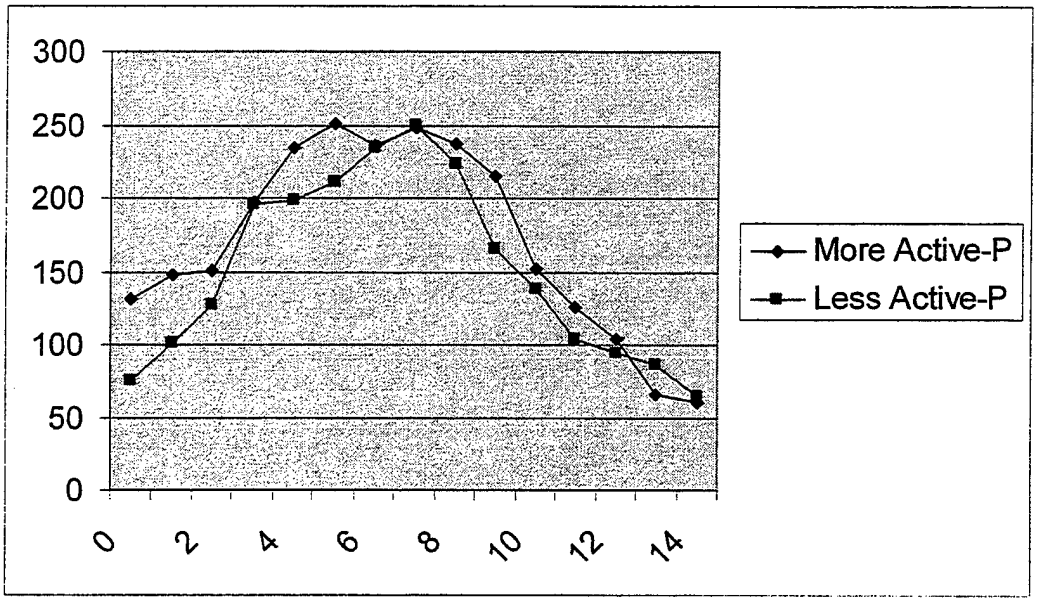


Figure 2 shows the average progesterone levels from the midcycle day until the end of the luteal phase. The more active twin had somewhat higher values during this period.

**Figure 2: Mean progesterone levels by menstrual cycle day during the luteal phase for 24 pairs with ovular cycles by activity level of twin.**



**6) Key Research Accomplishments**

- We have demonstrated that women are able to collect the saliva samples as requested and mail them to the Laboratory for analysis.
- Some difficulties in the collection of samples were found as well as in the useability of the samples once collected. Additional efforts need to be made in instruction and compensation to assure a higher compliance.
- Preliminary analyses have indicated that average daily luteal phase estrogen levels were slightly lower in the more active twin, whereas no difference was found in follicular phase estrogens. Progesterone levels, however, tended to be higher in the more active twin.

**7) Reportable Outcomes**

Hamilton, Ann, Lipson, Susan, Shames, Lisa, and Ellison, Peter , The Effect of Physical Activity on Menstrual Cycle Estrogens: A Study of Identical Twins, poster presented at the Era of Hope Meeting, Atlanta, Georgia, June, 2000.

A student interested in exercise physiology received training in research on this grant as she contacted the twins and conducted the screening interviews. At Dr. Ellison's Laboratory, a graduate student participated in the estradiol assays. A graduate student in biostatistics has assisted Dr. Hamilton in developing an index to determine the more active twin based on the main questionnaire and on the daily logs. Reliability between the two methods has been assessed.

Several publications are being planned including:

1) Non-invasive methods of obtaining estrogen levels from a complete menstrual cycle
2) Reliability of multiple assessments of physical activity levels
3) Effect of moderate physical activity on menstrual cycle estrogens

**8) Conclusions**

There is a suggestion that lower average daily luteal phase estrogens may be related to higher activity level in these identical pairs, although results were not significant after removing pairs with anovular cycles. Since more exposure to E2 occurs during the luteal phase, the reduction in exposure due to exercise may be significant over many years. The findings have implications for the influence of moderate exercise on ovarian function in non-athletes. More thorough understanding of the role of moderate exercise has important public health consequences and will be useful in determining strategies for reducing breast cancer risk.

We are continuing to analyze these results and will conduct multivariate analyses prior to the publication of results.

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